The EMBO Journal vol.8 no.8 pp.2387 - 2391, 1989

Apidaecins: antibacterial peptides from honeybees

Peter Casteels[1,2], Christophe Ampe[1,3], Frans Jacobs[2], Mark Vaeck[1] and Paul Tempst[1,4]

[1]Plant Genetic Systems NV, Plateaustraat 22 and [2]Department of Zoophysiology, Gent State University, Ledeganckstr. 35, B-9000 Gent, Belgium

[3]Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511, USA
[4]Present address: Howard Hughes Medical Institute and Department of Genetics, Harvard Medical School, Boston, MA 02115, USA

Communicated by M.Van Montagu

Although insects lack the basic entities of the vertebrate immune system, such as lymphocytes and immunoglobulins, they have developed alternative defence mechanisms against infections. Different types of peptide factors, exhibiting bactericidal activity, have been detected in some insect species. These humoral factors are induced upon infection. The present report describes the discovery of the apidaecins, isolated from lymph fluid of the honeybee (*Apis mellifera*). The apidaecins represent a new family of inducible peptide antibiotics with the following basic structure: GNNRP($^{V}/_{I}$)YIPQPRPPHPR($^{L}/_{I}$). These heat-stable, non-helical peptides are active against a wide range of plant-associated bacteria and some human pathogens, through a bacteriostatic rather than a lytic process. Chemically synthesized apidaecins display the same bactericidal activity as their natural counterparts. While only active antibacterial peptides are detectable in adult honeybee lymph, bee larvae contain considerable amounts of inactive precursor molecules.
Key words: antibacterial peptide/hemolymph proteins/insect immunity/solid-phase synthesis

Introduction

The presence of foreign material in the hemocoel (body cavity) of insects elicits a cellular and a humoral response which are detrimental to a wide variety of micro-organisms (reviewed by Dunn, 1986). The humoral immune response in insects involves the induction of several factors not present in naive (non-immune) lymph. Cecropins (mol. wt 4000), attacins (mol. wt 20 000), diptericins (mol. wt 8000) and insect defensin (sapecin) (mol. wt 4000) are the known bactericidal constituents of the humoral response in certain moths and flies (Dunn, 1986; Boman and Hultmark, 1987; Dimarcq *et al.*, 1988; Matsuyama and Natori, 1988; Lambert *et al.*, 1989). Previous studies on insect immunity were mainly performed on pre-imaginal stages of solitary insects. This report describes a new family of bactericidal peptides isolated from both larval and adult honeybees. We have named them apidaecins, from the Apidae, the family of insects to which honeybees belong.

Results

Isolation and characterization

Adult honeybees were injected in the body cavity with a sublethal dose of viable *Escherichia coli* cells; bactericidal activity appeared in lymph 8 h post-infection. After 24 h hemolymph samples were taken, cells were removed and the lymph fluid was tested for bactericidal activity. Control and immune lymph, both heat treated, were compared by reverse phase high performance liquid chromatography (RP-HPLC). At least nine factors were present in the immune lymph but not in untreated controls (Figure 1a and c). Lymph from bees injected with either India ink or latex beads had RP-HPLC patterns similar to lymph from *E.coli*-infected bees, excluding the possibility that the differential peaks are *E.coli*-derived material, and indicating that induction of these factors is not specific for the injected antigen. The results show that ink or latex challenge elicited a far lower bactericidal response than *E.coli* cells (data not shown). In other insects, the highest levels of bactericidal factors were also obtained with viable bacteria or their cell wall components (Dunn, 1986).

Immune components Apidaecin I and II were further purified by RP-HPLC and characterized. Their absorption characteristics and loss of activity upon treatment with trypsin or chymotrypsin indicated that they consist, at least in part, of polypeptide material. This was confirmed by amino acid composition and sequence analysis (Figure 2a). Apidaecin I and II were found to be basic peptides, 18 amino acids long, differing only at position 6 (Val versus Ile).

During sequencing of apidaecin I a small amount of Ile was detected at position 18, indicating that apidaecin I consists of two fractions (Ia and Ib) that could not be separated by RP-HPLC. However, component Ia eluted from the C18 column in the leading edge and Ib in the trailing edge of peak I; amino acid analysis of the first half of this peak showed a higher Ile content than of the second half and confirmed the sequencing results.

We estimate the ratio of apidaecin Ia to Ib to be 1:20 and that the total concentration of apidaecin (Ia + Ib + II) in immune bee lymph amounts of 50 nmol (100 μg) per ml. The NMR spectrum of apidaecin Ib was fully consistent with the data obtained from amino acid composition and sequence analysis (K.Hallinga, personal communication). The strong similarity among these three peptides suggests that different alleles are present in the population or that gene duplications may have given rise to their corresponding genes.

A search of the NBRF Protein Data Base (Washington, DC) revealed no significant similarities between apidaecins and bactericidal peptides from other insects or invertebrates nor with any other polypeptides. The other inducible factors involved in the immune response of honeybees are structurally unrelated to the apidaecins or other known peptide antibiotics and possess other activities than apidaecins (P.Casteels *et al.*, in preparation).

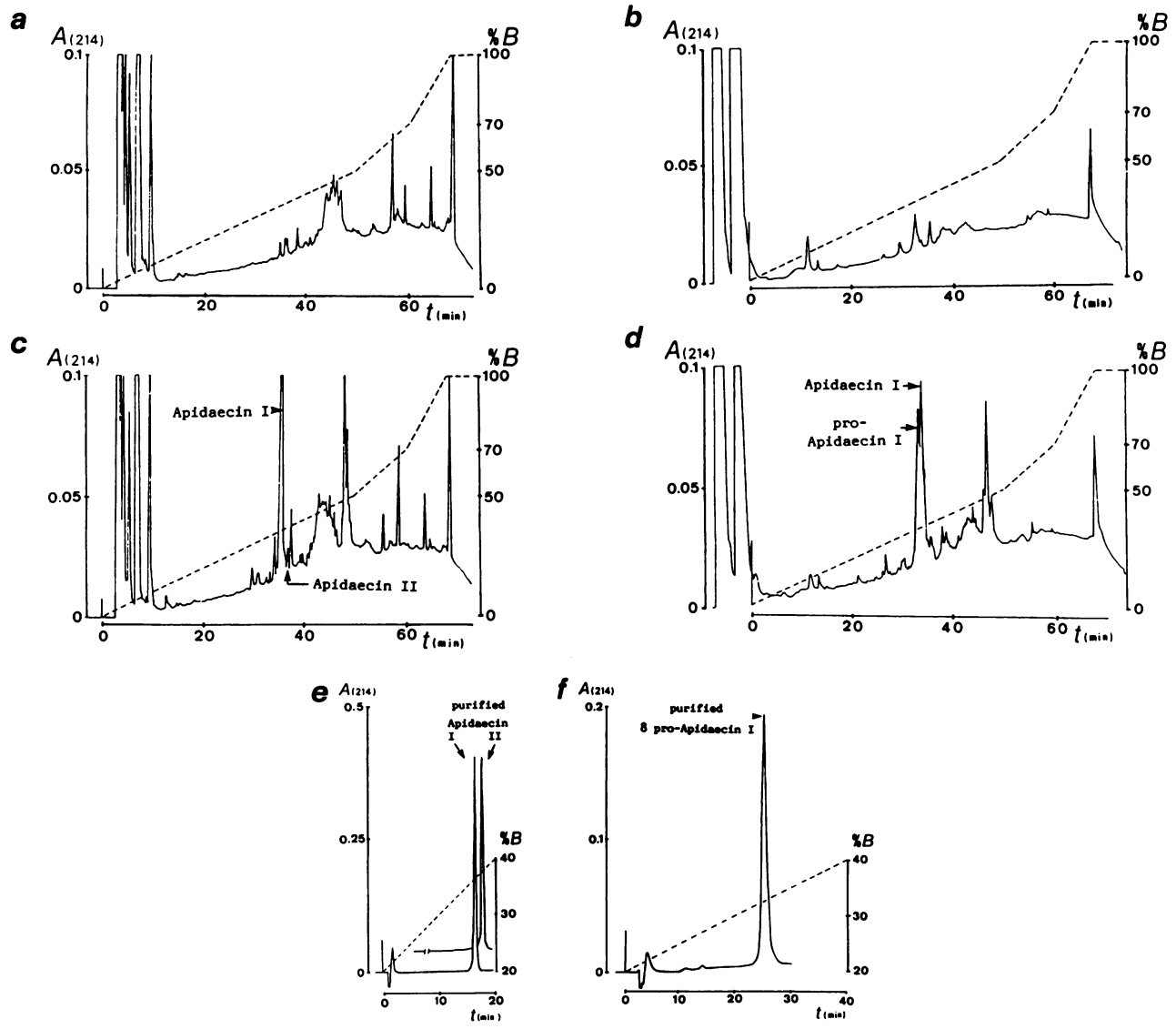

Fig. 1. HPLC analysis of control and immune honeybee lymph. **Panels a** and **b** show the HPLC patterns of lymph from, respectively, control (non-injected) adult and control larval honeybees; **panels c** and **d**, *E.coli*-induced lymph from, respectively, adult and larval bees. **Panel e**: natural apidaecins I and II purified on a C18 column. Apidaecin I consists of two fractions (Ia and Ib) that could not be separated by RP-HPLC (flow and gradients, see Materials and methods). **Panel f**: final purification of apidaecin precursor 8 pro-apidaecin I (see text) was done on a Vydac C4 column developed at a flow of 0.5 ml/min and a gradient of 20–40% B/40 min.

a) apidaecin

	1 5 10 15
Ia	G-N-N-R-P-V-Y-I-P-Q-P-R-P-P-H-P-R-I
Ib	G-N-N-R-P-V-Y-I-P-Q-P-R-P-P-H-P-R-L
II	G-N-N-R-P-I-Y-I-P-Q-P-R-P-P-H-P-R-L

b) 8 pro-apidaecin I $^{a}/_{b}$

E-A-K-P-E-A-K-P--G-N-N-R-P-V-Y-I-P-Q-P-R-P-P-H-P-R-$^{I}/_{L}$

c) pro-melittin

A-P-E-P-E-P-A-P-E-P-E-A-E-A-D-A-E-A-D-P-E-A--G-I-...-R-Q-Q-G

Fig. 2. (**a**) The amino acid sequence for apidaecins Ia, Ib and II, purified from immune lymph of honeybees (this study) and (**b**) precursor of apidaecins Ia and Ib isolated from honeybee larvae (this study) and (**c**) promelittin [sequence taken from (Kreil *et al.*, 1980)]. Every second residue in the precursor portions of the molecules (in italics) denotes the proposed successive processing sites. The arrows indicate the amino terminus of the mature peptides. The single amino acid differences are boxed.

Synthesis and bactericidal activity of apidaecins

The three apidaecins were chemically synthesized. Purified synthetic peptides were sequenced and compared with the natural bee peptides. No differences in NMR spectra or in chromatographic behaviour using RP-HPLC were detected between the synthetic peptides and their purified natural counterparts (data not shown).

We then evaluated the antibacterial activity of the natural and synthetic peptides against a wide variety of micro-organisms using a growth inhibition assay. The minimal inhibitory concentrations (MIC) of the apidaecins against 14 representative bacterial species are summarized in Table I, the MIC being the minimal concentration at which no growth could be detected in liquid culture. Human and animal pathogens (*Salmonella* and *Shigella* species) were very sensitive, whereas insect pathogens (*Bacillus thuringiensis, Serratia marcescens* and *Bacillus alvei*, a typical honeybee pathogen) were resistant. Furthermore, apidaecins were highly active against several plant-associated bacteria such as *Rhizobium* and *Agrobacterium* species and plant pathogenic bacteria belonging to *Erwinia* and *Pseudomonas* genera.

Comparison of the activity spectrum between natural apidaecin I and synthetic Ia, Ib and II revealed no significant differences, indicating that these peptides are functionally identical. A concentration of ~0.1 μg/ml was found to be sufficient to inhibit the development of several Gram(−) bacteria. Such a concentration corresponds to 0.1% of the concentration in immune bee lymph, clearly illustrating the effectiveness of the humoral response. Preliminary tests revealed no toxic effect of the peptides on either yeast or fungi.

The addition of 100 μg of synthetic apidaecins to a 1 ml liquid culture of 10^6 log phase *E.coli* cells resulted in a continuous (100%) inhibition of the cells as determined by plate count (data not shown). However, after immediate washing several times with salt buffer before plating out, some 20% of the cells recovered. Moreover, when cells were suspended in phosphate buffer in conditions that did not allow growth of the culture and treated with the same concentration of apidaecins, ~50% remained viable after washing and plating out. This suggests that apidaecins do not kill the bacteria immediately by lytic action but rather interfere with cell propagation. When apidaecins were added to an *E.coli* suspension (100 μg/ml), no lytic activity could be observed by measurement of the absorption (630 nm) nor with phase-contrast microscopy. Cecropins on the other hand were shown to display measurable lytic activity (Hultmark *et al.*, 1980). Our data indicate that apidaecins possess a high affinity for *E.coli* cells resulting in an immediate inhibition. 'Bound' apidaecins can be removed by washing, resulting in a recovery of viable cells.

To investigate whether apidaecins interact with phospholipid layers, their effect on a di-oleoyl phosphatidylserine monolayer was tested. No influence could be detected (data not shown) suggesting that these molecules do not act by disturbing phospholipid bilayers of the bacterial membrane.

Immune lymph of last instar larvae, from both drone and worker honeybees, contained several induced factors including reduced amounts of apidaecins Ia+b and II, but the RP-HPLC pattern also revealed an extra peak (pro-apidaecin I) eluting just before apidaecin I (Figure 1d). Fraction pro-apidaecin I was further purified by RP-HPLC using reduced flow-rates (Figure 1f). The predominant peptide was found to be an apidaecin precursor: EAKPEAKP-apidaecin I (8 pro-apidaecin I) (see Figure 2b); no detectable bactericidal activity could be associated with this purified precursor molecule. An analogous precursor sequence is present in promelittin (Figure 2c) which is processed by a dipeptidyl amino peptidase (DPAPase) activity (Kreil *et al.*, 1980). More examples of DPAPase processing have been reported for yeast α mating factor (Julius *et al.*, 1983) and cecropins A, B and D (Lidholm *et al.*, 1987). Similarly, processing of apidaecin precursors into mature peptides could occur through a stepwise cleavage of dipeptides ending in either a Pro or an Ala. We could not detect shorter precursor peptides; it is further unknown if this is the only processing event or whether it is preceded by another specific proteolytic cleavage of a prepro- into a pro-apidaecin.

Table I. Antibacterial activity of the apidaecin

Bacterial strains	Minimal inhibitory concentration (μg/ml) for apidaecin Ia, Ib and II			
	Ia	Ib	II	Ib*
Agrobacterium tumefaciens DSM 3129	0.2	0.2	0.2	1
Erwinia salicis NCPPB 2530	0.02	0.02	0.02	0.05
Escherichia coli NCTC 9001	0.1	0.1	0.2	0.5
Pseudomonas syringae pv. tomato NCPPB 1106	0.2	0.1	0.1	0.1
Rhizobium meliloti ZB 314	0.1	0.02	0.02	0.02
Salmonella Newport CD 94	0.2	0.2	0.2	0.5
Salmonella typhimurium ATCC 23565	0.1	0.1	0.1	0.1
Serratia marcescens ATCC 17991	+200	+200	+200	+200
Shigella flexneri CP 87	0.1	0.1	0.1	0.05
Corynebacterium insidiosum NCPPB 1109	50	50	100	100
Bacillus alvei LMG 6922	+200	+200	+200	+200
Bacillus megaterium QMB 1551	150	100	100	+200
Bacillus subtilis NRRL-B-237	+200	+200	+200	+200
Bacillus thuringiensis tenebrionis DSM 2803	+200	+200	+200	+200

The minimal concentrations for complete growth inhibition (μg/ml) were determined in liquid culture: 200 μl of medium (10% trypticase soy broth or nutrient broth) was inoculated with 10^4 log-phase bacteria. Peptide concentrations were 0/0.02/0.05/0.1/0.2/0.5/1/10/50/100/150/200. The values express μg/ml; when no inhibition was found the maximal values are given (+200). All peptides are chemically synthesized except for Ib* which was purified from immune honeybee lymph and contains ~5% Ia.

Discussion

We describe the structural properties and biological activities of apidaecins, a new family of peptide antibiotics purified from immune honeybee lymph. Apidaecins are the major humoral components induced in honeybee lymph upon bacterial infection. The induction is aspecific since it can also be provoked by injection of latex particles and India ink. Three closely related peptides, apidaecin Ia, Ib and II, were identified. These cationic peptides are highly stable at low pH (2) and high temperature (100°C). The presence of six prolines may contribute to this stability. Although apidaecins have some properties, such as heat stability and basic character, in common with cecropins no sequence similarity with these or other known peptides was found. The three apidaecins have an almost identical primary structure with differences in only a few residues. Apidaecins have mol. wts of 2100, making them the smallest immune peptides so far purified from insects. The exceptionally high proline content (33%) of these biologically active peptides is, to our knowledge, unique. Moreover, the high Pro content of the apidaecins theoretically excludes the peptide assuming a helical structure. Such a structure has been shown to be essential for the bacteriolytic activity of cecropins and several other peptide antibiotics (Lee *et al.*, 1986). The viability of *E.coli* cells treated with apidaecins under growth-limiting conditions is significantly higher compared with growing cells.

Our data also suggest that apidaecins do not disturb cellular membranes. Consequently, it is reasonable to postulate that the apidaecins may have a bacteriostatic rather than a bacteriolytic effect as was suggested for attacins (Engström *et al.*, 1984).

The immediate effect of apidaecins contrasts with the rather slow action of attacins; after addition of the latter the decrease in growth rate of an *E.coli* culture was only detectable at 1–2 h (Engström *et al.*, 1984). Magainins (Zasloff, 1987), cecropins and melittin (Steiner *et al.*, 1981) possess lytic activity and were found to perturb cellular membrane structures.

Apidaecins are highly active against Gram negative bacteria. All three different apidaecins showed comparable activities towards the bacteria tested.

The marked susceptibility of enteric and plant pathogenic bacteria suggests that honeybees have developed a defense system specifically targetted towards the micro-organisms they frequently encounter.

The lower level of mature apidaecins, together with the presence of incompletely processed, inactive precursors, in the lymph of bee larvae clearly suggests a lower dipeptidyl amino peptidase activity in larval lymph. Differences between the immune response of imaginal and pre-imaginal stages may be related to the social behaviour allowing bee-larvae, unlike larvae of solitary insects, to grow up in a more protected environment.

The lower concentration of active antibacterial factors in larval lymph may impair the effectiveness of their immune response. However, the larvae are fed by workerbees; the latter filter the food in the proventriculus (Verbeke *et al.*, 1984) and thereby reduce the risk of infection of the pre-imaginal stages.

The strong antibacterial action of apidaecins and their apparent non-toxicity towards eukaryotic cells promises possible applications as antibiotic compounds in the treatment of bacterial infections in animals or man.

The isolation and the characterization of the apidaecins now makes it possible to synthesize genes coding for these peptides. Transfer and expression of such genes in plants may result in the generation of transgenic crop varieties which are resistant against plant pathogenic bacteria.

Materials and methods

Induction and purification of antibacterial peptides

Adult and larval honeybees were injected with 5×10^4 viable *E.coli* (NCTC 9001) cells suspended in 1 μl phosphate-buffered saline (PBS: 0.15 M, pH 7.2). One day after injection the insects were bled by puncturing the abdomen with a glass capillary. The collected hemolymph (2–4 μl) was pooled in ice-cooled tubes containing a few crystals of phenylthiourea to prevent melanization of the samples. Hemocytes were centrifuged (10 000 *g* for 10 min) and the lymph was heat-treated (100°C, 5 min). The precipitate was spun down and the clear supernatant was acidified by addition of an equal volume of 0.1% trifluoroacetic acid (TFA). Samples (20 μl for analytical chromatography, Figure 1) of diluted lymph were taken for RP-HPLC analysis using an ABI 150A system (Applied Biosystems Inc., Ramsey, NJ) with a Vydac C4 (214TP54) analytical column (The separations group, Hesperia, CA). Solvent A was 0.1% TFA (pH 2) and solvent B: 70% acetonitrile (MeCN) in A. Fractions were eluted at 1 ml/min, except for Figure 1f, with a three-step linear gradient: 0–50% B/50 min, 50–70% B/10 min, 70–100% B/8 min (68 min total time). UV detection was done at 214 nm.

All differential peaks between control and immune lymph, including peaks apidaecin I and apidaecin II, were collected and further purified on Vydac C18 (218TP54) and diphenyl (219TP54) analytical columns. Collected fractions were lyophilized and redissolved in water before being tested for biological activity.

Following this procedure 80–120 μg apidaecin was routinely purified from a batch of 500 bees. This material was used for the determination of the primary structure and for screening some 100 micro-organisms on their susceptibility.

Primary structure determination

The sequences were determined using an ABI 470A (Applied Biosystems Inc., Foster City, CA) automated gas-phase sequencer (Hewick *et al.*, 1981) and an ABI 120A on line phenylthiohydantoin (PTH) amino acid analyzer. Stepwise liberated PTH-amino acids were quantified with a D2000 chromato-integrater (Hitachi). Apidaecin I(a+b) and 8 pro-apidaecin I(a+b) all showed a single sequence except for their C-terminal residue where a Leu/Ile (20:1) mixture was detected. The amino acid compositions, analyzed using both post-column *o*-phtaldialadehyde derivatization (Benson and Hare, 1975) and pre-column phenylisothiocyanate derivatization (Bidlingmeyer *et al.*, 1984) after 24 h and 72 h total hydrolysis (four analyses per sample), were in perfect agreement with the sequencing results.

Solid-phase synthesis of apidaecins

Chemical peptide synthesis was performed with an automated peptide synthesizer, model 430A (Applied Biosystems Inc.) t-Butyloxycarbonyl (tBoc)-N^{α}-protected, L-configuration amino acids were coupled sequentially to tBoc-Leu (or Ile)-OCH_2-phenylacetoamido methyl-(polystyrene resin) (Mitchell *et al.*, 1978). All reagents and solvents were from Applied Biosystems (ABI); the standard ABI synthesis protocol (STD1RR) was used.

After completion of the synthesis, the N^{α}-tBoc group was removed with 65% TFA and 0.5 g of the neutralized and dried peptide resin was cleaved for 1 h at −4°C with a mixture of 0.5 ml anisole and 4.5 ml of freshly distilled, anhydrous HF.

After removal of the HF under vacuum, the peptide was precipitated and extensively washed with diethyl ether, dissolved in 10 ml of 10% acetic acid and lyophilized. Purification was done on an ABI 151A semi-preparative HPLC system with a Vydac C4 column (1 × 25 cm). Purified material was analyzed on HPLC and by sequencing.

Acknowledgements

We thank Mr Frederik Leyns, Mr José Van Damme, Mr Richard Cook and Mr Gary Gryan for technical assistance, Dr Rudy Demel for doing monolayer experiments and Drs Jean Swings, Jan Leemans, Enno Krebbers,

George Church and Marc Van Montagu for helpful discussions. P.C. was supported by an IWONL (Belgium) graduate fellowship.

References

Benson,J.R. and Hare,P.E. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 619–622.

Bidlingmeyer,B.A., Cohen,J.A. and Tarvin,T.L. (1984) *J. Chromatogr.*, **336**, 93–104.

Boman,H.G. and Hultmark,D. (1987) *Annu. Rev. Microbiol.*, **41**, 103–126.

Dimarcq,J.-L., Keppi,E., Dunbar,B., Lambert,J., Reichhart,J.-M., Hoffmann,D., Rankine,S.M., Fothergill,J.E. and Hoffmann,J.A. (1988) *Eur. J. Biochem.*, **171**, 17–22.

Dunn,P.E. (1986) *Annu. Rev. Entomol.*, **31**, 321–339.

Engström,P., Carlsson,A., Engström,A., Tao,Z. and Bennich,H. (1984) *EMBO J.*, **3**, 3347–3351.

Hewick,R.M., Hunkapiller,M.W., Hood,L.E. and Dreyer,W.J. (1981) *J. Biol. Chem.*, **256**, 7990–7997.

Hultmark,D., Steiner,H., Rasmuson,T. and Boman,H.G. (1980) *Eur. J. Biochem.*, **106**, 7–16.

Julius,D., Blair,L., Brake,A., Sprague,G. and Thorner,J. (1983) *Cell*, **32**, 839–852.

Kreil,G., Haiml,L. and Suchaneck,G. (1980) *Eur. J. Biochem.*, **111**, 49–58.

Lambert,J., Keppi,E., Dimarcq,J.-L., Wicker,C., Reichhart,J.-M., Dunbar,B., Lepage,P., Van Dorsselaer,A., Hoffmann,J., Fothergill,J. and Hoffmann,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 262–266.

Lee,S., Mihara,H., Aoyagi,H., Kato,T., Izumiya,N. and Yamasaki,N. (1986) *Biochim. Biophys. Acta*, **862**, 211–219.

Lidholm,D.-A., Gudmundsson,G.H., Xanthopoulos,K.G. and Boman,H.G. (1987) *FEBS Lett.*, **226**, 8–12.

Matsuyama,K. and Natori,K. (1988) *J. Biol. Chem.*, **263**, 17112–17116.

Mitchell,A.R., Kent,S.B.H., Engelhardt,M. and Merrifield,R.B. (1978) *J. Org. Chem.*, **43**, 2845.

Steiner,H., Hultmark,D., Engström,A., Bennich,H. and Boman,H.G. (1981) *Nature*, **292**, 246–248.

Verbeke,M., Jacobs,F.J. and De Rycke,P.H. (1984) *Am. Bee J.*, **124**, 468–470.

Zasloff,M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5449–5453.

Received on April 10, 1989; revised on April 28, 1989